



Symbiotic microbiome and metabolism profiles reveal the effects of induction by oysters on the metamorphosis of the carnivorous gastropod *Rapana venosa*



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ABSTRACT

Most marine mollusks have a pelagic larval phase, and they need to undergo metamorphosis to develop into adults. Metamorphosis is affected by many factors, including abiotic factors such as temperature, salinity and illumination as well as biological factors such as food and microorganisms. In our previous study, we found that the metamorphosis of *Rapana venosa* requires induction by juvenile oysters, which are the food source of *R. venosa*. However, the regulatory mechanism of this induction is largely unknown. In the present study, we evaluated the impacts of induction by juvenile oysters on competent larvae of *R. venosa*. Competent larvae were experimentally divided into two pools, and scallop shells without juvenile oysters and scallop shells with juvenile oysters were added for 2 h and 12 h to monitor alterations in critical gene expression, symbiotic microbiota and metabolomic responses. The carboxypeptidase gene was increased while the cellulase gene was decreased, which may mean that the food habit transition was induced by juvenile oysters. Meanwhile, critical genes in the neuroendocrine system were also significantly altered in juvenile oysters. Furthermore, dramatic changes in the symbiotic microbiota and metabolism profiles were observed, with many of them associated with the digestive system and neuroendocrine system. In conclusion, juveniles as food resources may induce metamorphosis in *R. venosa* by regulating the neuroendocrine system and promoting the development of the digestive system and changes in digestive enzymes. This study may provide evidence that induction by juvenile oysters can promote food habit transition and metamorphosis in *R. venosa* by regulating the metabolome and microbiome and further altering the digestive and neuroendocrine systems of *R. venosa*, which expands our understanding of the regulatory mechanism of metamorphosis in *R. venosa*. However, further studies are needed to explore the specific substance inducing metamorphosis released by juvenile oysters.

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1. Introduction

Metamorphosis is required for most marine invertebrates to complete their biphasic life cycle, which is crucial in their development [1,2]. However, extensive morphological and physiological changes occur during metamorphosis and are accompanied by high mortality [1,3]; therefore, metamorphosis controls molluscan population dynamics. Metamorphosis is not a simple spontaneous development process. In addition to developing to a certain stage

(competent larvae), having an appropriate inducer is necessary. At present, research on the metamorphosis regulation mechanism has mainly focused on bivalve mollusks and herbivorous gastropods, such as juvenile oysters and abalones, which have been studied deeply and for which perfect regulatory models have been formulated [4–6]. However, few studies about the metamorphosis of carnivorous gastropods have been conducted. The mechanisms of metamorphosis in carnivorous gastropods are more complex and dramatically different from the metamorphosis of lifelong herbivorous gastropods because they need to undergo a food habit transition from herbivorous to carnivorous during metamorphosis. If proper food cannot be provided in time, they will face death from starvation or cannibalism [7]. Therefore, the metamorphosis of carnivorous gastropods is accompanied by higher mortality than that of lifelong herbivorous gastropods.

The veined rapa whelk (*Rapana venosa*), a typical carnivorous gastropod, is an economically important gastropod in China due to its high economic and medicinal value [7–10]. Unfortunately, *R. venosa* is highly threatened, and its population has dramatically declined in recent years in China [11,12]. Although artificial aquaculture started early in the 1990s [11,13], the large-scale culture of this species is seriously limited by its high mortality during metamorphosis. On the other hand, as a carnivorous gastropod, *R. venosa* is an invasive species in many areas in the world [14], which causes serious damage to bivalve molluscan resources in many places, and controlling its population is urgently necessary. Therefore, its metamorphic mechanism must be studied not only to protect domestic shellfish resources, but also to avoid the damage caused by the invasion of *R. venosa*.

Our previous study found that oysters can significantly improve the metamorphosis rate of larvae, and juvenile oysters less than 3 cm in size had a better induction effect, especially the oysters which were just metamorphosis [15] (Fig. S1). Additionally, we also found a “collection effect” of *R. venosa* around juvenile oyster reefs in the wild, which may further indicate a close ecological relationship between *R. venosa* and juvenile oysters. Bather [16] showed that the presence of conspecific adults or useful food sources could trigger metamorphosis in marine invertebrates. Hadfield and Paul [17] found that the metamorphosis of abalone larvae was induced by some red algae, which contain some analogs of GABA [18,19]. However, the specific induction effect of juvenile oysters on *R. venosa* is unknown and of great significance to reveal the regulatory mechanism of metamorphosis in *R. venosa*.

In our previous studies, we found that the digestive system and the neuroendocrine system were dramatically changed during metamorphosis [20,21]. Critical digestive enzymes, such as *carboxypeptidase* and *cellulase*, were significantly changed during metamorphosis, which reflects the food habit transition in the metamorphosis of *R. venosa*. Additionally, the expression of some key genes in the neuroendocrine system, such as 5-hydroxytryptamine receptor (5-HT receptor) and nitric oxide synthase (NOS), was also significantly changed and has also been found to play an important role in metamorphosis in many invertebrates. Furthermore, cholecystokinin (CCK), the key hormone regulating the digestive system [22–24], and its receptor were also significantly changed during metamorphosis. We also found that CCK can induce alterations in digestive enzymes in competent larvae of *R. venosa* [25]. A previous study reported that feeding plants to grass carp in advance can promote the development of individuals by regulating some critical hormones and changing the activity of digestive enzymes [26]. Therefore, whether oysters as food resources induce metamorphosis in *R. venosa* by regulating the neuroendocrine system and promoting the development of the digestive system and changes in digestive enzymes needs further investigation.

Growing evidence indicates that the symbiotic microbiota plays a key role in regulating host energy metabolism and neurotransmitter synthesis by releasing small molecules of metabolites and acting on the nervous system [27,28]. To adapt to the environment or the physiological condition of the host, the composition and metabolic products of the symbiotic microbiota can change adaptively and directly or indirectly regulate the important life course of the host [27,29]. Meanwhile, growing evidence supports that the symbiotic microbiota plays a key role in regulating host digestive and metabolic functions [28]. Cavalcanti et al. [30] reviewed the vital impact of bacteria on the metamorphosis of invertebrates, and we also found dramatic alterations in symbiotic microbiota during metamorphosis in *R. venosa* [31]. Therefore, we hypothesize that symbiotic microbiota may play an important role in regulating the alteration of the digestive system and neuroendocrine system in the metamorphosis mentioned above. Metabolomic profiling is powerful for identifying metabolic changes due to environmental chemical exposure and screening molecular biomarkers [32]. Currently, an increasing number of studies have combined 16S rRNA amplicon sequencing and metabolomic methods to investigate the correlations between symbiotic microbiota changes [33].

In the present study, we aimed to evaluate the effect of induction by juvenile oysters on competent larvae of *R. venosa*. Based on previous studies of the metamorphosis of *R. venosa*, we hypothesized that induction by juvenile oysters regulates the metamorphosis of *R. venosa* by affecting the interaction of the digestive system and neuroendocrine system of the host with symbiotic microbiota. To explore these changes, we used juveniles to induce competent larvae and investigated the alteration of the expression of critical genes in the digestive system and neuroendocrine system, including carboxypeptidase, cellulase, 5-HT receptor, NOS and CCK receptor. Furthermore, 16S rRNA amplicon sequencing and metabolomics profiles were used to identify the symbiotic microbiota alterations and metabolic responses of the host to induction by juvenile oysters. The data will reveal the effect of induction by juvenile oysters on competent larvae of *R. venosa* and may provide new insight into the regulatory mechanism of metamorphosis in carnivorous gastropods from the perspective of the neuroendocrine and digestive systems.

2. Materials and methods

2.1. Sample collection

Culturing of parent whelks, spawning and larval rearing were performed according to Yang et al. [11]. Planktonic larvae were cultured in $3.5 \times 5.2 \times 1.5$ m cement pools at 24.6–25.6 °C and a density of 0.1 ind/mL. *Isochrysis galbana*, *Chlorella vulgaris* and *Platymonas subcordiformis* were mixed as a diet for the planktonic larvae fed three times daily at 5.0×10^4 cells/mL each time. The larvae were observed under a microscope to ensure developmental synchrony such that over 90% of the larvae in each sample were at the same developmental stage. Until the larvae reached the four-spiral stage (shell height > 1,200 μm, competent larvae), we divided the pool of larvae into two pools as evenly as possible for the juvenile oyster induction assays. Here, the oyster species we used was *Crassostrea gigas*. The scallop shells with oysters were acquired according to Zhou et al. [34], when the proportion of eyespot larvae (shell height 270–320 μm) of oyster reached 20%–30%, the attachments (scallop shells) were put into pools to collect oyster larvae, until the juvenile oysters on scallop shells were reached 1000 μm. All of the scallop shells with oysters provided by Blue Ocean Co. Limited (Laizhou, Shandong, China). The following controls and treatments were included in the assays: (1) seawater with scallop shells without juvenile oysters (control group, C)

and (2) seawater with juvenile scallop shells with juvenile oysters (juvenile oyster induction group, O). We randomly collected 15 samples from each pool at 2 h (early stage, e) and 12 h (later stage, l) after treatment and washed them three times with PBS. Sixty samples were obtained from four groups, including the control group at 2 h (Ce) and 12 h (Cl) and the treatment group at 2 h (Oe) and 12 h (Ol), and each sample contained 50 larvae (Fig. S2). These samples were used to analyze the mRNA expression of critical genes related to metamorphosis, including carboxypeptidase, cellulase, 5-HT receptor, NOS and CCK receptor, microbial community and host metabolomics. All samples were stored at -80°C until use.

2.2. Critical gene expression analysis

Three samples from each group were homogenized, and total RNA was extracted using the DNA/RNA/Protein Coextraction Kit (DP423, TIANGEN Biotech Co., Ltd., Beijing, China) following the manufacturer's protocol. The quality was determined by agarose gel electrophoresis and a Nanodrop spectrophotometer (Thermo Fisher Scientific, America). First-strand cDNA for qRT-PCR was synthesized using the Prime Script™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan). Primers for qRT-PCR were designed based on the full-length cDNA sequences of the carboxypeptidase, cellulase, 5-HT receptor, NOS and CCK receptor genes (Table S1), which are dramatically shifted during metamorphosis in *R. venosa* [20,25,35]. The 60S ribosomal protein L28 (RL28-F, RL28-R) was used as a housekeeping gene for signal normalization within the experiment [36]. SYBR® Premix ExTaq™ (TaKaRa, Japan) was used with an Eppendorf Mastercycler® ep Realplex (Eppendorf, Hamburg, Germany) for qRT-PCR analysis. Relative gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.

2.3. Microbial community analysis

Five samples from each group were homogenized, and genomic DNA or PCR amplification was extracted using the E.Z.N.A.® soil DNA Kit (Omega Biotek, Norcross, GA, U.S.) according to the manufacturer's instructions. The concentration and purity of DNA were checked on a 1% agarose gel and determined with a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). PCR amplification of the 16S rRNA gene was performed before sequencing, and the V3-V4 regions of the bacterial 16S rRNA gene were amplified with the primers 338F (5'-ACTCTACGGGAGG CAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The PCR mixtures contained 4 μl $5 \times$ TransStart FastPfu buffer, 2 μl 2.5 mM dNTPs, 0.8 μl forward primer (5 μM), 0.8 μl reverse primer (5 μM), 0.4 μl TransStart FastPfu DNA Polymerase, 10 ng template DNA, and finally up to 20 μl ddH₂O. The PCR amplification of the 16S rRNA gene was performed as follows: initial denaturation at 95 $^{\circ}\text{C}$ for 3 min, followed by 27 cycles of denaturing at 95 $^{\circ}\text{C}$ for 30 s, annealing at 55 $^{\circ}\text{C}$ for 30 s and extension at 72 $^{\circ}\text{C}$ for 45 s, a single extension at 72 $^{\circ}\text{C}$ for 10 min, and a final extension at 4 $^{\circ}\text{C}$. After amplification, PCR products were detected on 2% (w/v) agarose gels, purified by the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and finally quantified by QuantiFluor™-ST (Promega, USA). The size of each amplicon was no less than 550 bp, and the amplicons were sequenced (2 \times 250) by Majorbio Co., Ltd. in Shanghai using the MiSeq PE300 platform (Illumina, USA).

The raw 16S rRNA gene sequencing reads were demultiplexed and quality-filtered using USEARCH 7.1 (<http://drive5.com/uparse/>) [37], of which sequences containing > 3 identical consecutive bases or with quality scores < 20 were eliminated. Clean reads were obtained by removing chimeric sequences (http://www.drive5.com/usearch/manual/chimera_formation.html) [38].

Operational taxonomic units (OTUs) were selected (equivalent to 97% sequence similarity) and clustered using UPARSE 7.1 software with the default parameters (<http://drive5.com/uparse/>) [39]. Subsequently, the effective sequences were aligned against the SILVA database (<http://www.arb-silva.de>) and then identified by the Ribosomal Database Project (RDP, <http://rdp.cme.msu.edu/>) Bayesian classifier with a 70% threshold. The OTU abundance information was normalized using a standard sequence number corresponding to the sample with the fewest sequences, and it was used for the subsequent analysis.

A Venn diagram that was used to count the number of unique and shared OTUs in multiple samples was constructed using the Draw Venn Diagram online tool (<http://bioinformatics.psb.ugent.be/webtools/Venn/>). Barplot and Pieplot analyses of the community composition were constructed by the Vegan package in R 2.4 at the OTU and genus levels, respectively. The alpha diversity indices of the symbiotic microbiota, Shannon index, Simpson index, ACE index and Chao index were calculated by Mothur (<http://www.mothur.org/wiki>). A principal coordinate analysis (PCoA) was performed using QIIME 1.7.011 based on weighted UniFrac distances to investigate beta diversity, which was further tested through the differences in the microbiota communities among groups with ANOSIM ($P < 0.05$). The network analysis for connections among bacterial groups in *Rapana venosa* with or without induction by juvenile oysters on the genus level was based on the Spearman coefficient, and species whose absolute value of the correlation coefficient was ≥ 0.6 were selected and plotted in Cytoscape software ($P < 0.05$). Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict the functions of microbiota by predicting the Kyoto Encyclopedia of Genes and Genomes (KEGG) categories and functional enzymes [40].

2.4. Metabolomic analysis

Five samples from each group were used for the metabolomic analysis, and they were accurately weighed and extracted using a 400 μl methanol:water (4:1, v/v) solution. The extraction was settled at -20°C and treated with a Wonbio-96c high-throughput tissue crusher (Shanghai Wanbo Biotechnology Co., Ltd.) at 50 Hz for 6 min, followed by vortexing for 30 s and ultrasonication at 40 kHz for 30 min at 5 $^{\circ}\text{C}$. The samples were placed at -20°C for 30 min to precipitate proteins. After centrifugation at 13,000 \times g at 4 $^{\circ}\text{C}$ for 15 min, the supernatant was carefully transferred to sample vials for LC-MS/MS analysis. The mass spectrometric data were collected using a Thermo UHPLC-Q Exactive Mass Spectrometer equipped with an electrospray ionization (ESI) source operating in either positive or negative ion mode. As a part of the system conditioning and quality control process, a pooled quality control sample (QC) was prepared by mixing equal volumes of all samples.

After the analyses, the raw data were imported into Progenesis QI 2.3 (Nonlinear Dynamics, Waters, USA) for peak detection and alignment and then filtered. The minimum metabolite values were imputed for specific samples in which the metabolite levels fell below the lower limit of quantitation, and each metabolic feature was normalized by the sum. The internal standard was used for data QC (reproducibility). Metabolic features with a relative standard deviation (RSD) of QC > 30% were discarded. Following normalization procedures and imputation, a statistical analysis was performed on log-transformed data to identify significant differences in metabolite levels between comparable groups. The mass spectra of these metabolic features were identified by using the accurate mass, MS/MS fragment spectra and isotope ratio differences and searching reliable biochemical databases, such as the

Human Metabolome Database (HMDB) (<http://www.hmdb.ca/>) and Metlin database (<https://metlin.scripps.edu/>).

A multivariate statistical analysis was performed using ropls (Version 1.6.2, <http://bioconductor.org/packages/release/bioc/html/ropls.html>). A partial least squares discriminate analysis (PLS-DA) was used to determine the metabolic changes between comparable groups. A Venn diagram was constructed using the Draw Venn Diagram online tool (<http://bioinformatics.psb.ugent.be/webtools/Venn/>). The P values were estimated with a paired Student's t -test on a single-dimensional statistical analysis, and a heatmap was generated to illustrate the alterations of significantly changed metabolites between groups using the R package (VIP value > 1 , $P < 0.05$). The KEGG database (<http://www.genome.jp/kegg/>) was used to analyze the functional enrichment of the differential metabolites between groups. These metabolites can be classified according to the pathways in which they are involved or the functions they perform. A Procrustes analysis was employed to reveal the correlation and variation between symbiotic microbiota and differential metabolites, and it was conducted by calculating the Monte Carlo p -value and M2. A P value < 0.01 indicated that the trend of symbiotic microbiota and metabolite expression level was very significantly consistent among different groups; M2: The sum of squares of deviations analyzed by Procrustes. The smaller the value, the higher the degree of association between the two data points. The M2 statistics and their significance test P values provide an overall measure of consistency between the two data sets. Partial data were analyzed on the free online platform Majorbio Cloud Platform (www.majorbio.com).

2.5. Data analysis

Levene's test was used to test the equality of variances in the analysis of critical gene expression and the alpha diversity indices, and significant differences between groups were analyzed by Student's t -test. All statistical analyses were performed using SPSS 19.0. A P value < 0.05 indicated that the difference was significant.

3. Results

3.1. Changes in critical gene expression induced by juvenile oysters

To assess the alteration of the digestive system and neuroendocrine system in competent juvenile larvae induced by juvenile oysters, the mRNA expression of carboxypeptidase, cellulase, 5-HT receptor, and NOS and CCK receptor was detected (Fig. 1). The expression of the carboxypeptidase gene was increased in both the early and later stages in competent larvae induced by juvenile oysters, while cellulase expression was decreased in the later stage. The expression of the 5-HT receptor was significantly increased, while the expression of the NOS and CCK receptor was significantly decreased in juvenile oysters ($P < 0.05$) (Fig. 1), which may mean that induction by juvenile oysters can significantly affect competent larvae of *R. venosa*.

3.2. Symbiotic microbiota alteration induced by juvenile oysters

16S rRNA amplicon sequencing analysis based on high-throughput sequencing was conducted to determine the changes in the symbiotic microbiota community in competent *R. venosa* induced by juvenile oysters. Quality and chimera filtration of the raw data produced 929141 high-quality sequences from 17 samples (the amplified products of OI_1, Oe_4, and Oe_5 were not up to standard), with an average of 54,655 reads, and raw data were submitted to the sequence read archive (SRA) with project accession number PRJNA715398. When the alignment was performed

at an average length of 416 bp, OTUs were clustered at a 3% distance, and each of these OTUs represented a unique phylotype. Finally, 2821 OTUs were obtained, and the OTUs were classified into 41 phyla and 877 genera (Table S2).

The Venn diagram showed that 579 OTUs were shared among the four groups, and 169, 323, 103 and 392 OTUs were unique to groups Ce, Cl, Oe and OI, respectively (Fig. 2 A). The top 9 dominant OTUs in the four groups are shown in Fig. 2 B (low-abundance OTUs (< 0.05) were merged into "others"); 7 of them belonged to Alphaproteobacteria, Gammaproteobacteria, and Proteobacteria, and 2 belonged to Cyanobacteria. Proteobacteria and Cyanobacteria were the most abundant phyla in the symbiotic microbiota in *R. venosa*, consistent with our previous studies [31]. At the genus level, *norank_f_norank_o_Chloroplast* was the most dominant genus in the Ce and Cl groups (control), and its relative abundance was more than 45%; *norank_f_norank_o_Chloroplast* was still the most dominant genus in the Oe group, but its relative abundance decreased to 37.47%. However, the abundance of *norank_f_norank_o_Chloroplast* was only 1.38% in OI, while *Ralstonia* became the dominant genus (32.72%) (Fig. 2 C).

To illustrate the diversity and richness of the symbiotic microbiota in each sample, the Shannon index, Simpson index, ACE index and Chao index were calculated and are shown in the boxplot graph. The Shannon index and Simpson index are often used to quantify the diversity of microbes, and we found that the Shannon index of Cl was significantly higher than that of Ce, while the Simpson index was the opposite, and no difference was found between the groups induced by juvenile oysters (Oe and OI) and the control (Ce and Cl) (Fig. 3 A, B). The richness of each sample was calculated via the ACE index and Chao index. The ACE index and Chao index of Cl were significantly higher than those of Ce. Additionally, the ACE indices of Oe and OI were significantly lower than those of Ce and Cl, respectively (Fig. 3 C, D). Meanwhile, the PCoA showed that the four groups were significantly divided, and the Cl and OI groups were significantly separated from the other groups ($P < 0.05$, ANOSIM in Table S5). The Ce and Oe groups were relatively close (Fig. 3 E), which suggested that both the short development period (10 h) and the induction by oysters had significant effects on the structure of the symbiotic microflora of the larvae, and the former had been reported in our previous study [31].

To identify the highly connected microbial community and to predict the key species and their interactions, we conducted a single-factor correlation network analysis at the genus level. The results showed that *Tepidibacter*, *Roseovarius*, *norank_f_norank_o_Gaiellales*, *norank_f_Hyphomicrobiaceae* and *Oceanirhabdus* were most highly correlated with others in the community structure (Fig. 4 A). We further investigated the significant differences between Ce and Cl and between Cl and OI to reveal the effect of development time and oysters. Among the top 10 abundant genera that had significant differences between Ce and Cl (Fig. 4 B), the abundances of *Vibrio* and *Endozoicomonas* were significantly higher in the Ce group than in the Cl group, while *Bacillus*, *Rhodococcus*, *Roseovarius* and the other 5 genera had significantly higher abundances in the Cl group than in the Ce group. Among the top 10 abundant genera that had significant differences between Cl and OI, the abundances of *Ralstonia*, *Mesorhizobium*, *Endozoicomonas*, *Burkholderia-Caballeronia-Paraburkholderia*, *Rhodococcus* and *Sphingomonas* were significantly higher in the OI group than in the Cl group (Fig. 4 C), while *Chloroplast*, *Bacillus*, *unclassified_c_Gammaproteobacteria* and *Mycobacterium* had significantly higher abundances in the Cl group than in the OI group. *Roseovarius*, belonging to Rhodobacteraceae, has been highly correlated with other species and significant differences among groups, and it may be a key species in the metamorphosis of *R. venosa* induced by juvenile oysters.

The changes in the putative functions of the symbiotic microbiota of *R. venosa* were examined by predicting the 16S rRNA

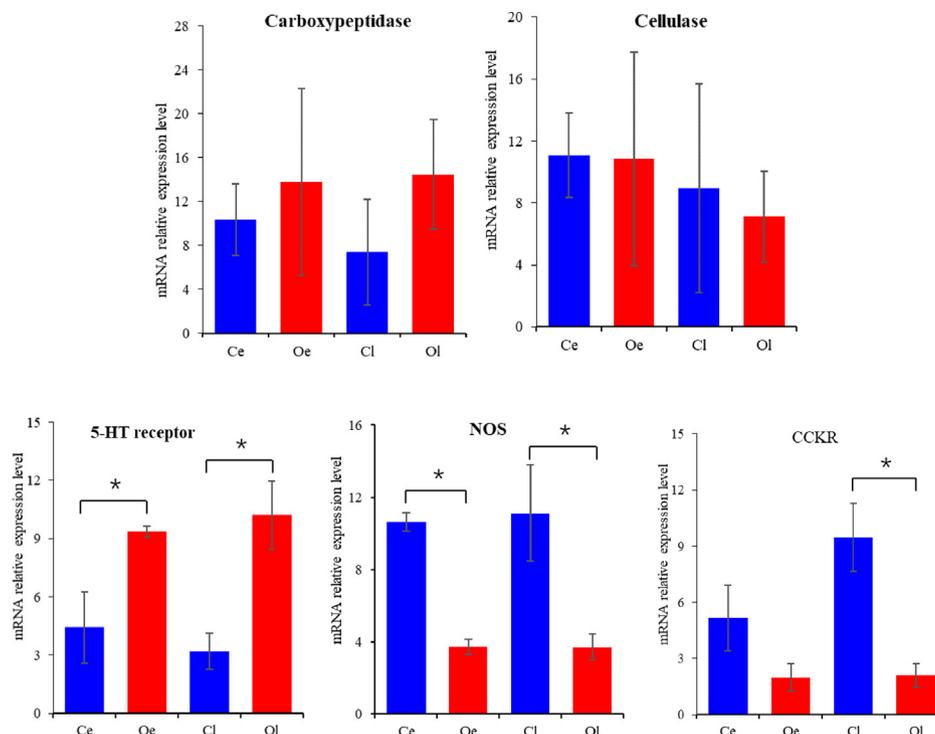


Fig. 1. qRT-PCR analysis of carboxypeptidase, cellulase, 5-HT receptor, NOS and CCK receptor mRNA expression in competent larvae induced by oyster (mean \pm SE, $n = 3$). Different superscript letters indicate significant differences ($P < 0.05$, the results of statistical test is in Table S3).

amplicon using PICRUSt. The metagenomic prediction showed the 30 most enriched pathways, which included transporters, ATP-binding cassette (ABC) transporters, DNA repair and recombination proteins, two-component systems, purine metabolism, arginine and proline metabolism, and so on. Compared with Ce, all 30 pathways were more abundant in Cl (Fig. 5 A). Comparing Ol with Cl, the abundance of 14 pathways was upregulated, including “ABC transporters”, “transcription factors”, and “fatty acid metabolism”. Sixteen pathways were downregulated, including “arginine and proline metabolism”, “purine metabolism”, and “methane metabolism” (Fig. 5 B).

3.3. Metabolomic alteration induced by juvenile oysters

A metabolomic analysis was conducted to address the alterations in metabolic profiles in competent larvae of *R. venosa* induced by juvenile oysters and development. The PLS-DA plot based on all metabolite abundances showed clear separation of metabolic profiles in the four groups; Ce and Oe were farther apart, while Cl and Ol were closer together (VIP value > 1 , $P < 0.05$) (Fig. 6 A). To explore the effect of induction by oysters on larvae, we emphatically analyzed the shared differential metabolites between the Cl and Ol groups and the Ce and Oe groups. Meanwhile, we emphatically analyzed the shared differential metabolites between the Oe and Ol groups and the Ce and Cl groups to explore the effect of brief development. The Venn diagram showed that 61 differential metabolites were shared between Oe vs. Ce and Ol vs. Cl (Fig. 6 B), and 47 differential metabolites were shared between Cl vs. Ce and Ol vs. Oe (Fig. 6 C). Additionally, the hierarchical clustering heatmap showed 61 metabolites in Ol vs. Cl (19 upregulated metabolites and 42 downregulated metabolites) and 47 metabolites in Cl vs. Ce (30 upregulated metabolites and 17 downregulated metabolites). Among them, the levels of Pseudomone, Aldosine and Cyclo (L-prolyl-L-leucyl) were significantly higher in Ol, while the levels of Sterebin A, L-arginium and Pisumic were

lower in Ol than in Cl. (Fig. 6 D); and the levels of L-arginine, L-histidine and L-lysine were significantly higher in Cl, while the levels of pseudomone, deoxyinosine and inosine so on were lower in Ol than in Cl. (Fig. 6 E). Differential metabolites were further analyzed by KEGG annotation (VIP value > 1 , $P < 0.05$). The results showed that “arginine biosynthesis” was the most significantly enriched pathway according to the differential metabolite shared between Cl vs. Ce and Ol vs. Oe ($P < 0.001$) and had the highest enrichment ratio (Fig. 7 A). “Purine metabolism” was the most significantly enriched pathway according to the differential metabolites shared between Ol vs. Cl and Oe vs. Ce ($P < 0.001$) (Fig. 7 B).

3.4. Association between the altered metabolites and symbiotic microbiota

Induction by oysters and development induced significant alterations in the metabolome and symbiotic microbiota. To investigate the potential association between metabolites and symbiotic microbiota genera, Procrustes analysis showed that apparent correlations were identified between the altered metabolites and perturbed bacteria families with Monte Carlo P value = $0.005 < 0.01$, $M^2 = 0.673$ (Fig. 8). This may suggest that the metabolites and symbiotic microbiota interact, influence each other, and jointly regulate the food habit transition and metamorphosis of *R. venosa* [32,33].

4. Discussion

Previous studies have shown that symbiotic microbiota and metabolic profiles are dramatically altered with larval development during metamorphosis [21,31]. Here, we attempted to determine the main induction effect of juvenile oysters on *R. venosa* and reveal the regulatory mechanism of metamorphosis induced by juvenile oysters from the perspective of the digestive system and

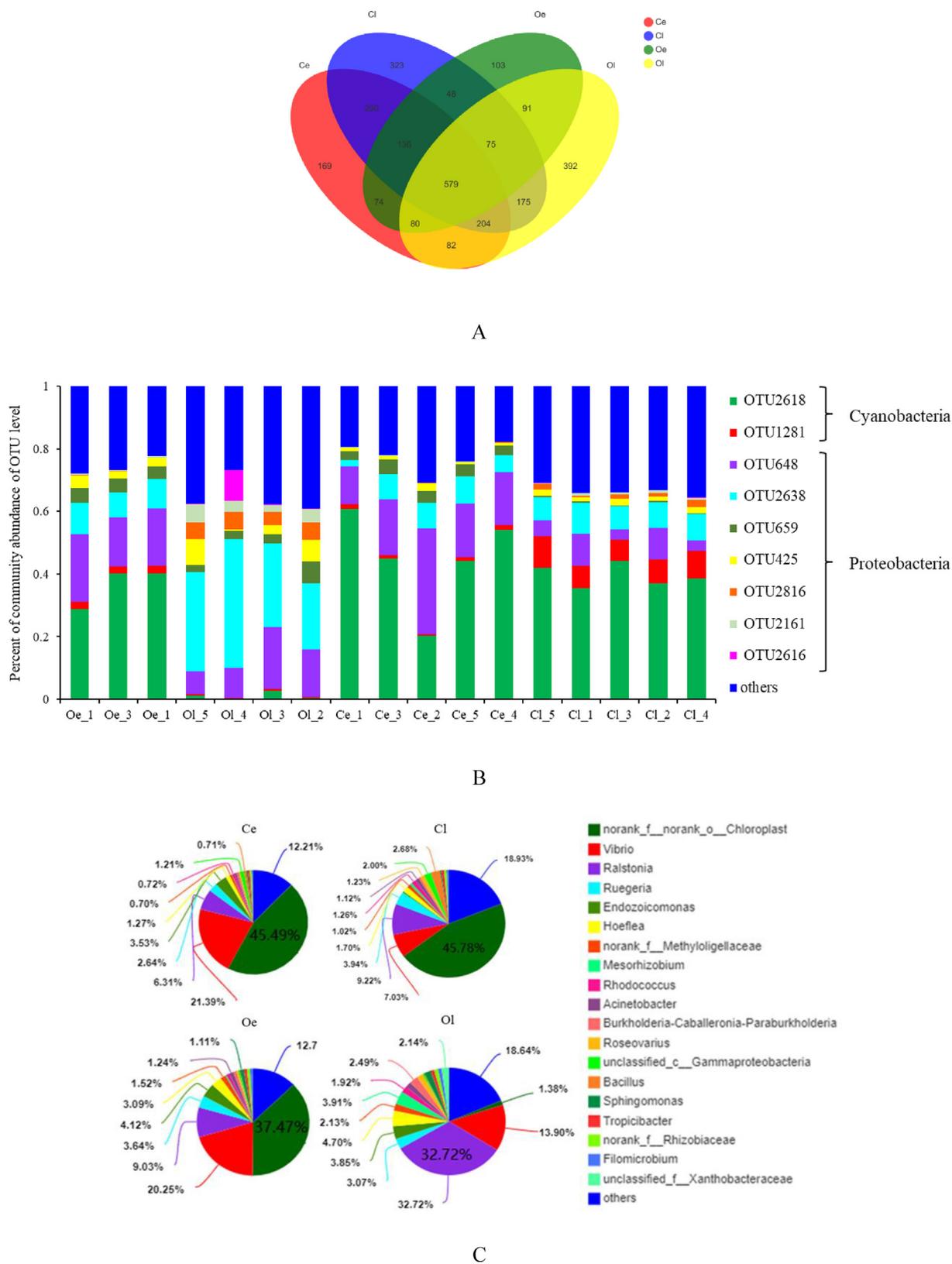


Fig. 2. The community composition of symbiotic microbiota in *Rapana venosa*. (A) Venn diagram of OTUs in groups Ce, Cl, Oe, OI. (B) Barplot of bacterial abundance at the OTU level (Low abundance OTUs (<0.05) were merged into “others”). (C) Pieplot of bacterial abundance at the genus level (Low abundance genera (<0.05) were merged into “others”).

neuroendocrine system. For that, we determined the change in critical gene expression in the digestive system and neuroendocrine system, analyzed the symbiotic microbiota and metabolic

profile of *R. venosa* induced by juvenile oysters and further investigated the correlation between symbiotic microbiota and host metabolites. The results showed significant shifts in the gene

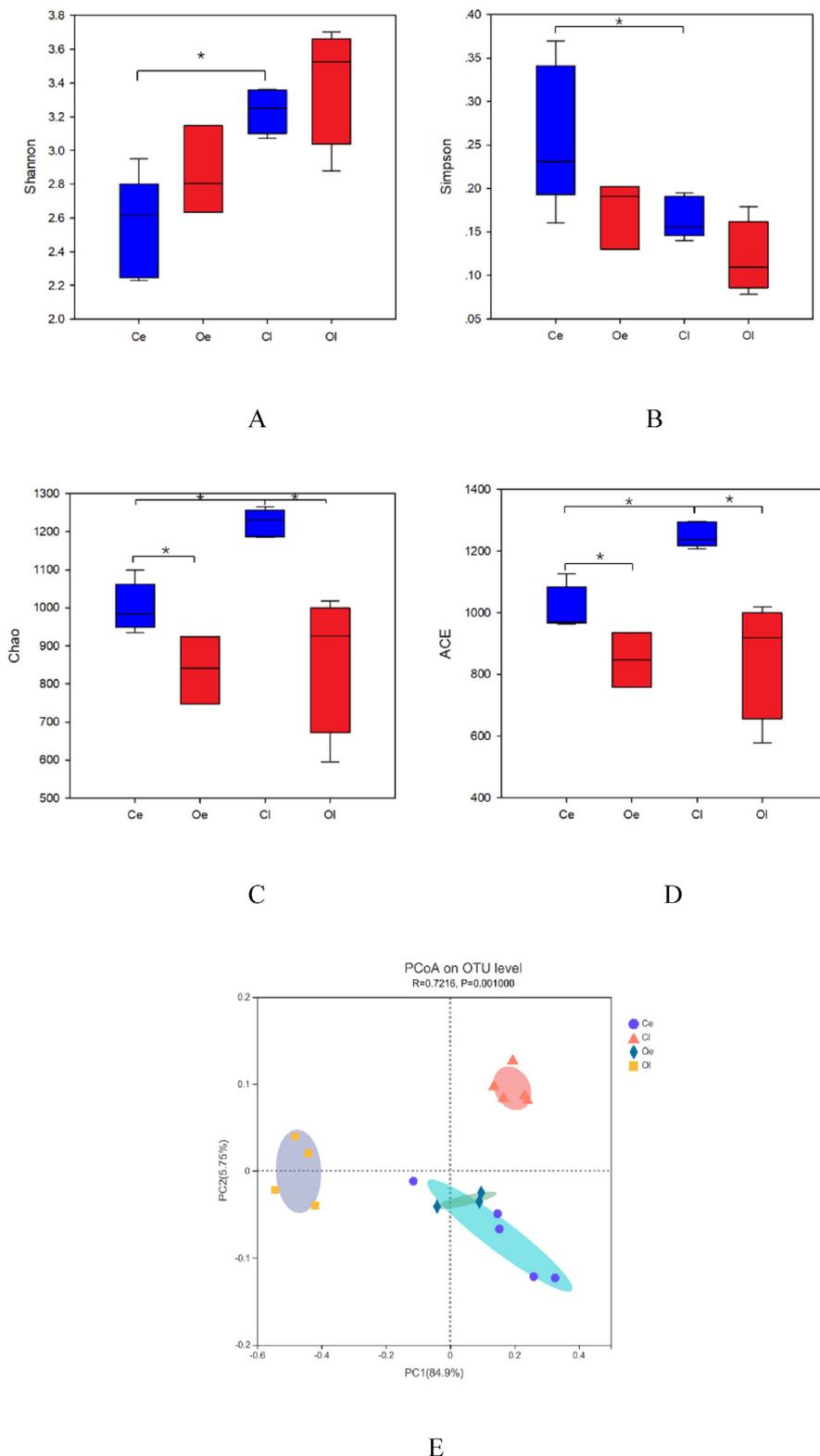


Fig. 3. Diversity analysis of symbiotic microbiota in *Rapana venosa* induced by oyster. α -diversity analysis includes (A) Shannon index, (B) Simpson index, (C) ACE index and (D) Chao index ($P < 0.05$, the results of statistical test is in table S4). β -diversity analysis includes (E) Principal coordinates analysis on OTU level (PCoA).

expression, symbiotic microbiota and metabolism profile of *R. venosa* induced by juvenile oysters, which may reflect a drastic impact of juvenile oysters on the competent larvae of *R. venosa*, and revealed dramatic changes in larvae during critical developmental periods.

4.1. Response of digestive system

In this study, induction by juvenile oyster increased the expression of carboxypeptidase while slightly decreasing the expression of cellulase (Fig. 1), which is consistent with the change in food

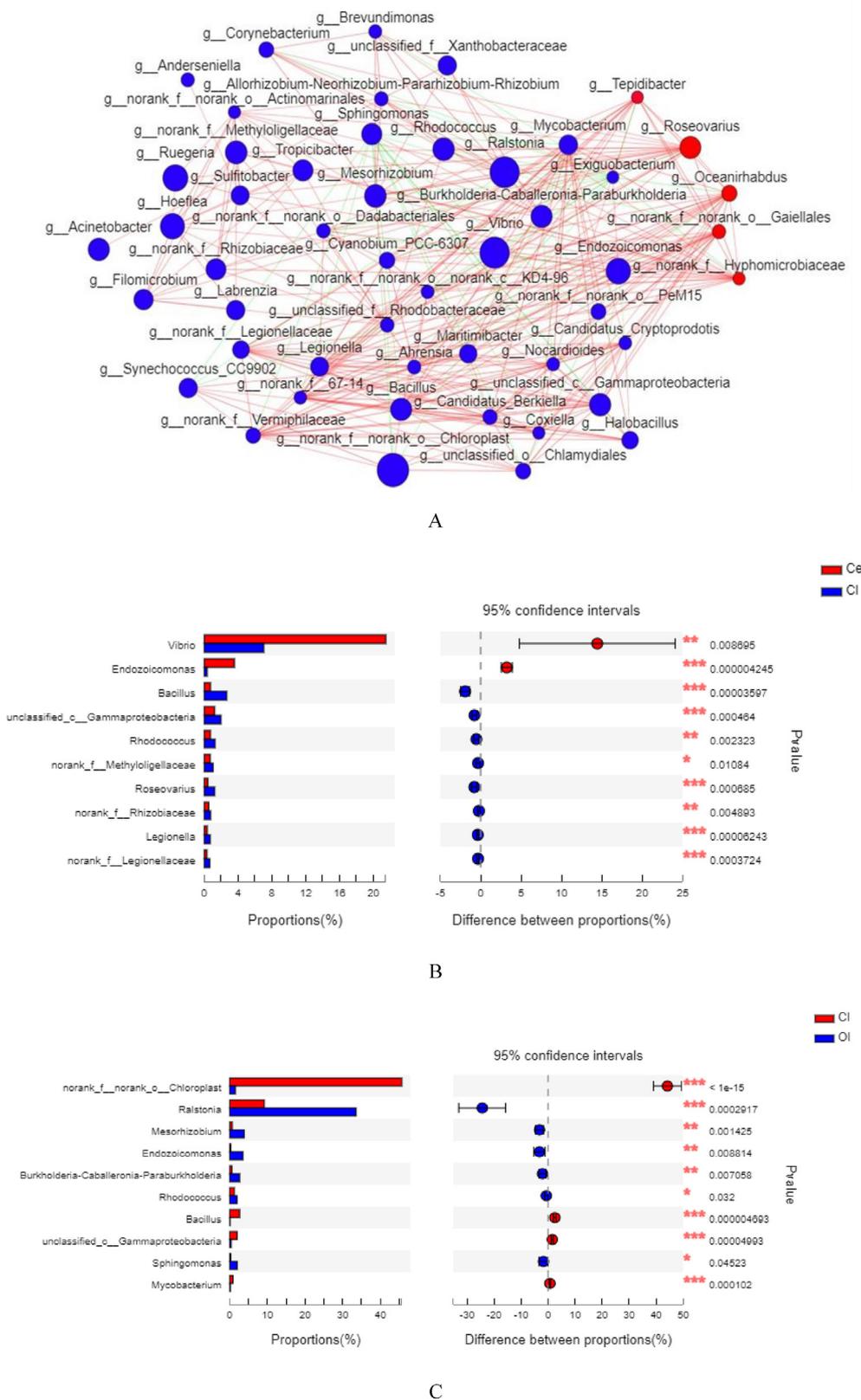


Fig. 4. Network analysis for connection among bacterial groups in *Rapana venosa* with or without the induction of oyster on genus (A). (Based on Spearman coefficient, species whose absolute value of correlation coefficient ≥ 0.6 were selected and plotted. The species with red nodes were in the top 5 of the central coefficient, and the species with red connector represented positive correlation and the species with green connector represented negative correlation, $P < 0.05$). Bacterial taxa on genus level differentially represented between two groups, in Ce vs Cl (B) and Cl vs OI (C).

habit transition during the metamorphosis of *R. venosa* [20,31,35]. Our results suggest that the digestive system of the *R. venosa* larvae responded to the induction by juvenile oysters and that digestive

enzyme expression changed to meet the needs of food habit transition and metamorphosis, which is consistent with a previous study in grass carp showing that feeding plants to juvenile grass

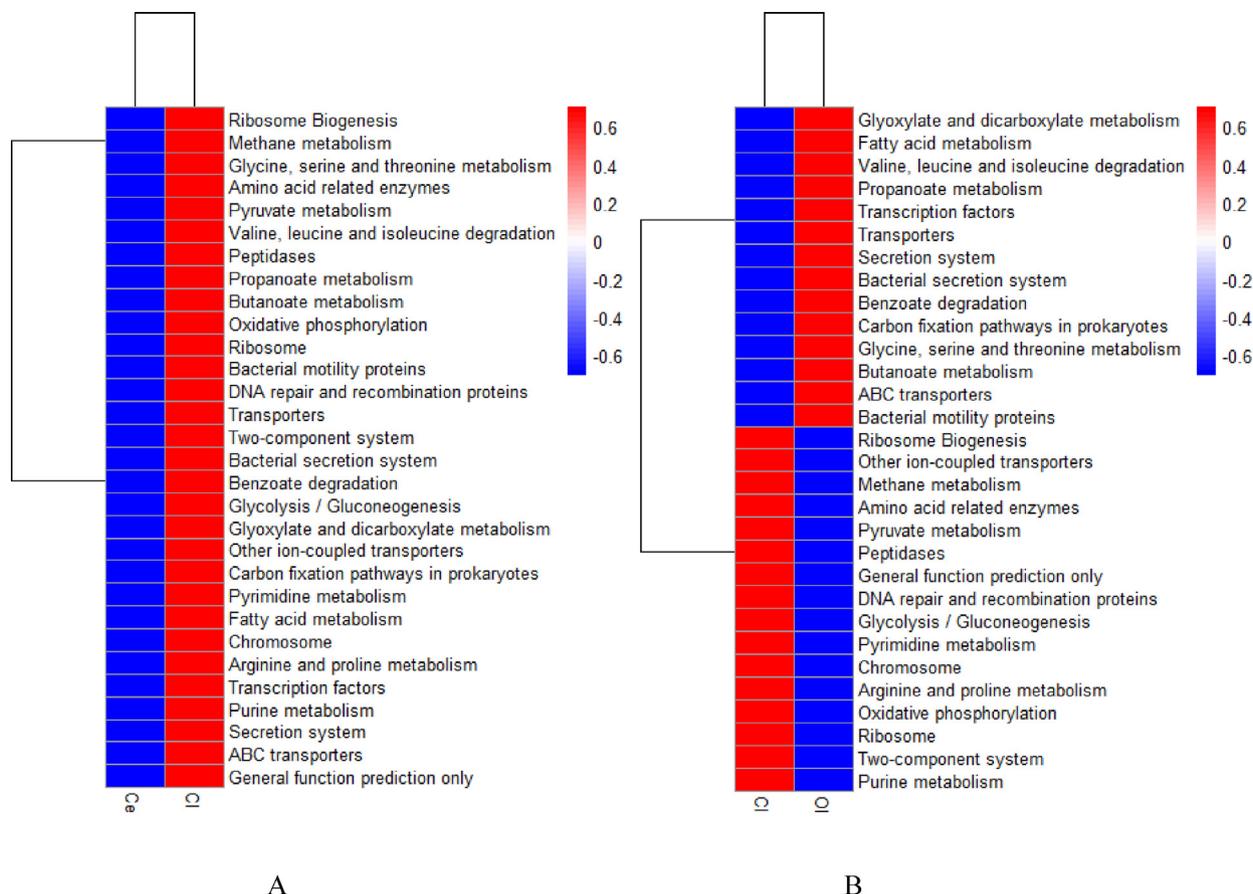


Fig. 5. Relative abundances of predicted functions of symbiotic microbiota in KEGG level 3, Ce vs Cl (A), Cl vs OI (B).

carp in advance can promote the development of its digestive system and change the activity of digestive enzymes [25]. Kinouchi et al. [24] indicated that large protein molecules and polysaccharides can stimulate the secretion of CCK and promote the development of the digestive system and the production of digestive enzymes. In the present study, we observed that the expression of the CCK receptor was significantly decreased under induction by juvenile oysters (Fig. 1); this may imply that CCK regulates food habit transition and metamorphosis by combining with the CCK receptor, which had been reported in a previous study [35].

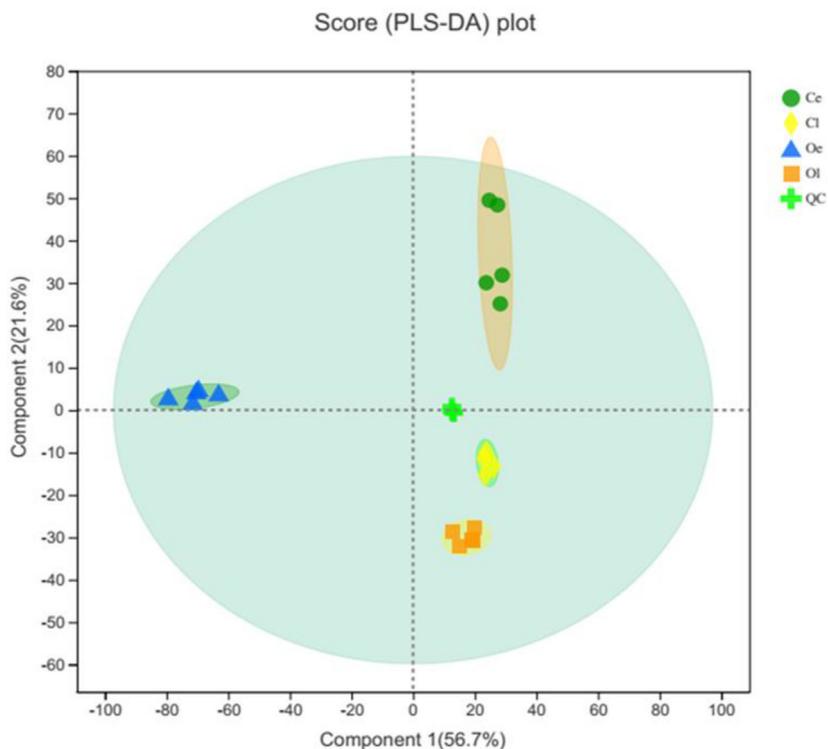
The microbiome plays an important role in the digestive system. Rhodobacteraceae has been reported to exist in the intestines of many aquatic organisms [41–43]; in addition, feeding with β-glucan can promote the growth of Rhodobacteraceae and activate the NF-κB signaling pathway via the PHB gene in Rhodobacteraceae in the intestine of *Apostichopus japonicus* [44,45]. In our results, the significant change and high correlation with other species may suggest that Rhodobacteraceae play an important role in food habit transition and metamorphosis induced by juvenile oysters. Meanwhile, a previous study indicated that the microflora of oyster soft tissue was dominated by bacteria affiliated with the family Rhodobacteraceae, which belong to Alphaproteobacteria [46,47]; this may further imply the impact of Rhodobacteraceae on the induction process between *R. venosa* and oyster.

4.2. Response of the neuroendocrine system

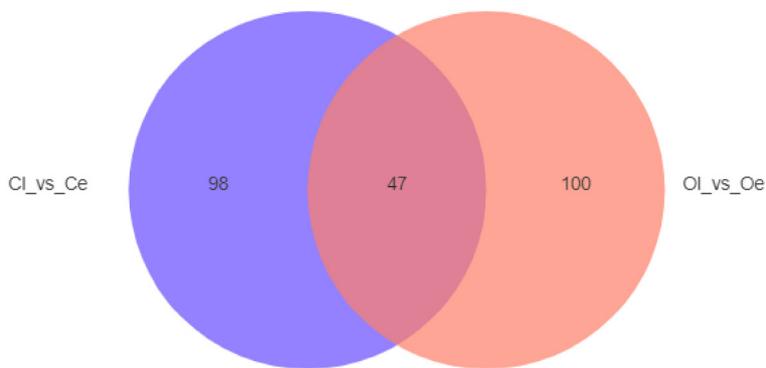
Correspondingly, the change in the expression of NOS further confirms the vital role of signal transduction pathways mediated by NO and NOS, which have been reported in many invertebrates [48–51]. Nobuo and Degnan [48] found that induction by coralline

algae *Amphiroa ephedraea* (food for larvae) made the expression of NOS change significantly in abalone larvae, and a similar result was observed in our study, which may further confirm the importance of NO and NOS in processing signals from inductive biomolecules to regulate the initiation of metamorphosis. However, in contrast to abalone, the NOS expression of *R. venosa* larvae was decreased around the time of acquiring competency as well as under the induction by juvenile oysters, which may suggest the different regulation of NOS in different species with different food habits (Fig. 1). Additionally, we found that the “arginine biosynthesis” pathway was significantly enriched in Cl vs. Ce and OI vs. Oe according to the differential metabolites, and the level of arginine (L-arginine or L-argininium) was higher in Cl than in Ce and OI (Fig. 6 D, E). Arginine is a necessary precursor for the synthesis of NO, a signaling molecule that plays a conserved role in regulating larval metamorphosis in a diverse range of marine invertebrates [51]. Studies on the larvae of snails, sea slug, and polychaetes have revealed that NO levels remain elevated when larvae are in the planktonic stage; however, once the larva encounters a metamorphic cue, the NO levels suddenly decrease. Therefore, researchers have shown that an elevated level of NO before encountering a metamorphic cue may enhance the probability of locating a suitable substratum by delaying metamorphosis and the dispersal capability of the larvae, thus increasing the gene flow between distant populations, which in turn enhances individual vigor [50]. Therefore, the increase in arginine and NOS in Cl may be used to increase the NO levels to delay metamorphosis before encountering the metamorphic cue (oyster) versus the decrease in OI.

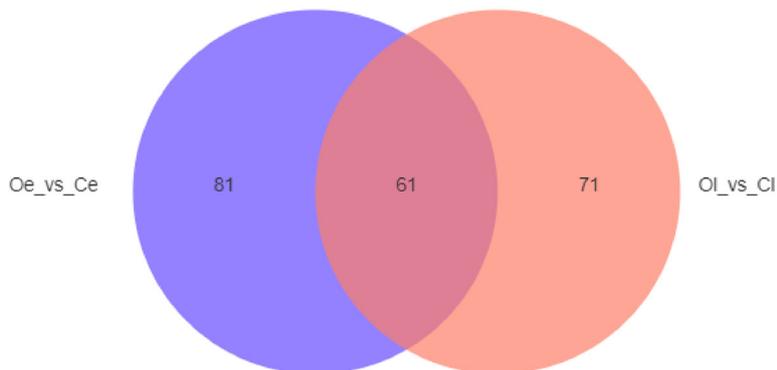
Interestingly, the KEGG analysis of the symbiotic microbiota showed that the “arginine and proline metabolism” pathway was also significantly more enriched in Cl than in Ce and less enriched



A

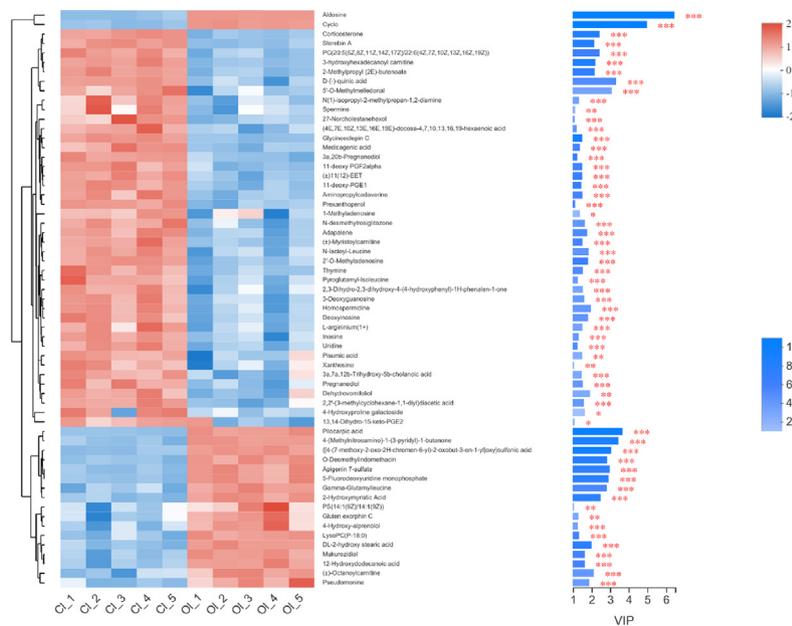


B

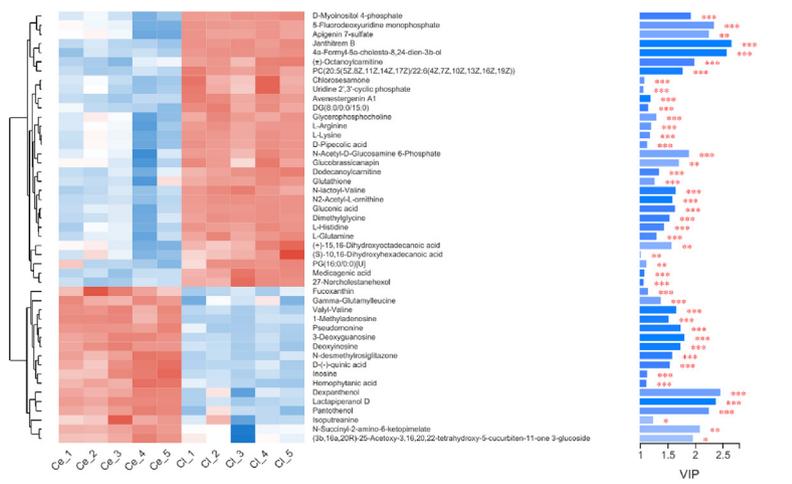


C

Fig. 6. Metabolic analysis in *Rapana venosa* induced by oyster and development. (A) Partial Least Squares Discriminant Analysis based all metabolites (PLS-DA). (B) and (C) Venn diagram of differential metabolites shared between Cl vs Ce and Ol vs Oe, and Oe vs Ce and Ol vs Cl. (D) and (E) Heat map shows the alteration patterns of significantly changed metabolites within Ol vs Cl and Cl vs Ce (VIP > 1).



D



E

Fig. 6 (continued)

in OI than in CI. Previous studies reported that the substrate for NO synthesis—arginine—cannot be biosynthesized but instead must be exogenously sourced in most invertebrates. Song et al. [51] found that the proteobacterial symbionts in sponge larvae were able to biosynthesize arginine, which was metabolized into NO by the host, regulating the metamorphosis of the host. This condition may also exist in the process of metamorphosis of *R. venosa*. Additionally, Proteobacteria was the dominant species and is known to be involved in the degradation of a variety of organic compounds (e.g., carbon, nitrogen, and sulfur) in aquatic ecosystems [52,53]. A previous study also indicated that most of the symbiotic microbiota related to nitrogen metabolism in sponges and corals belong to Proteobacteria and Cyanobacteria. For example, *Ralstonia* has been reported to be related to nitrogen metabolism and can use its denitrification pathway to detoxify NO (a plant defense signal). Detoxification may play synergistic roles in bacterial wilt virulence by converting the host's chemical weapon into an energy source, which is important for the pathogenesis of some *Ralstonia* strains in plant systems [54]. Therefore, we speculated that *Ralstonia* may promote the metamorphosis of *R. venosa* by reducing the con-

tent of NO. However, how induction by oysters increases the abundance of *Ralstonia* is unknown.

Leise et al. [49] indicated that exogenous NO can inhibit such serotonergically induced metamorphosis, while bath application or injection of exogenous 5-HT can initiate metamorphosis in competent larvae; furthermore, we found that in contrast to NOS, the expression of the 5-HT receptor was significantly increased by juvenile oysters (Fig. 1) [55,56]. Sahoo and Khandeparker [50] presented two possibilities: elevated NO reduces the serotonin level by inactivating tryptophan hydroxylase (TH) and/or converts serotonin into either 4-nitrososerotonin or 4-nitro-serotonin, rendering ineffective binding of modified serotonin with their receptors. However, once the larvae encounter a metamorphic cue, the NO level suddenly starts decreasing with a concomitant increase in serotonin production, which is consistent with the results of this study. Additionally, 5-HT has been indicated to be the key factor in signal transduction and metamorphosis initiation, and 5-HT and 5-HT receptor in the apical organ can promote metamorphosis by sensing external chemical signals [49]. In detail, the level of serotonin (5-HT) also increased after induction by juvenile oysters

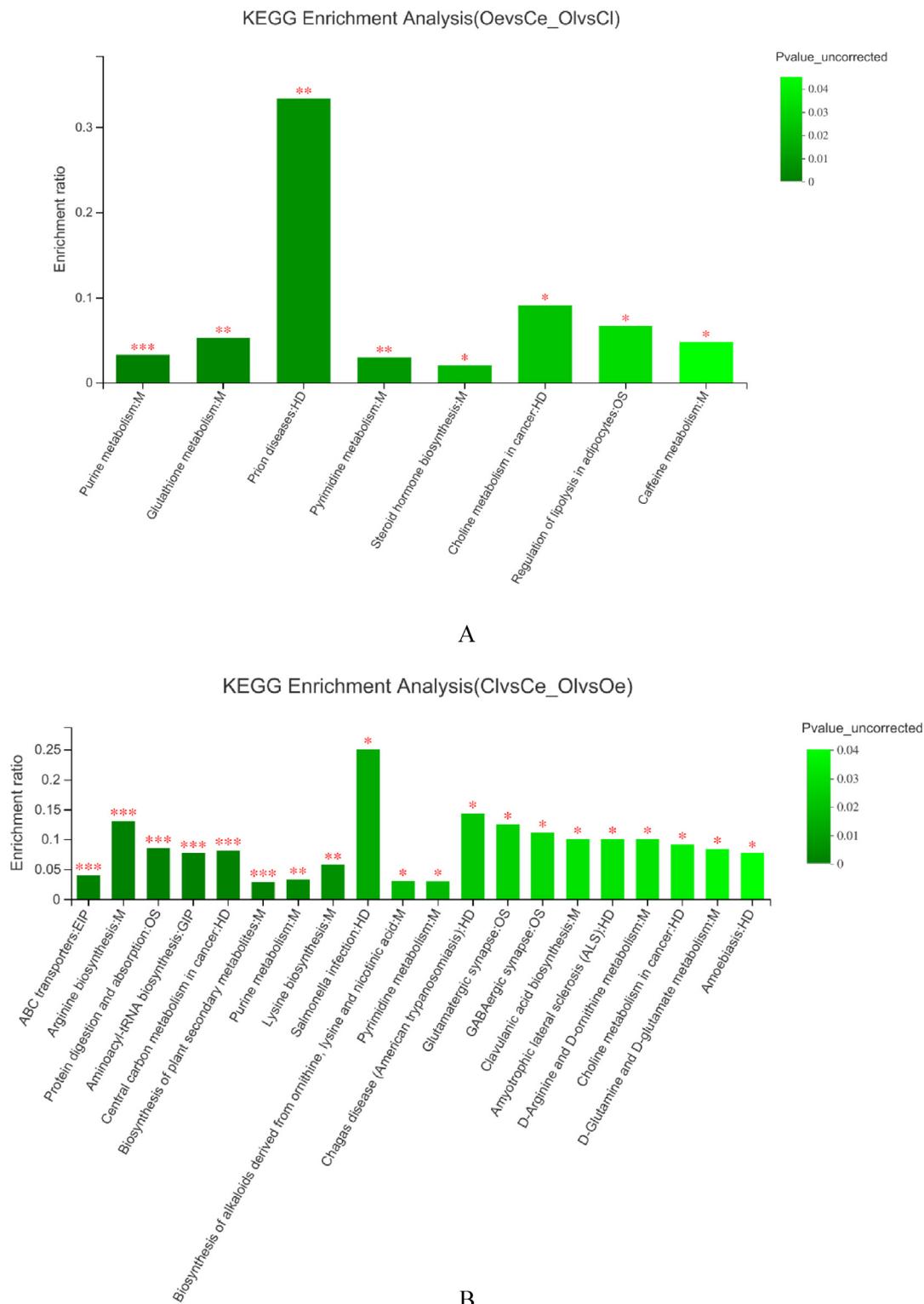


Fig. 7. Enriched KEGG pathways analysis of differential metabolites shared between Oe vs Ce and Ol vs Cl, and Cl vs Ce and Ol vs Oe (VIP > 1).

(Fig. S3 A), which is consistent with the change in expression of the 5-HT receptor. However, a previous study reported that the 5-HT receptor was downregulated after metamorphosis in *R. venosa* [57]. Therefore, we speculated that 5-HT and its receptor play an important but transient role in the food habit transition and initiation of metamorphosis induced by oysters in *R. venosa*. Additionally, the KEGG analysis showed that differential metabolites induced by juvenile oysters were significantly enriched in the serotonin receptor agonist/antagonist pathway (Fig. S3 B) [58].

4.3. Other responses

Furthermore, previous studies have shown that *Pseudomonas* can induce metamorphosis of the larvae of *Ciona intestinalis* by producing substances such as polysaccharides [59]. In the present study, we identified four OTUs belonging to *Pseudomonas* (Fig. S4), and their abundance was significantly higher in Ol than in the other three groups. Meanwhile, pseudomonine, a product of *Pseudomonas* [60], was also highly expressed in the Ol group

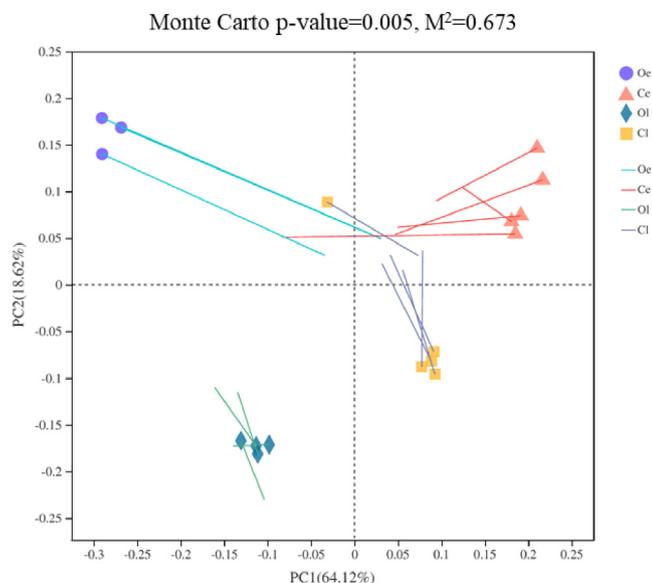


Fig. 8. Procrustes analysis. (The p -value < 0.01 indicated that the trend of symbiotic microbiota and metabolite expression level was very significantly consistent among different groups; M^2 : The sum of squares of deviations analyzed by Procrustes. The smaller the value, the higher the degree of association between the two data.)

(Fig. 6). This may suggest that the induction by juvenile oysters increased the abundance of *Pseudomonas*. However, the relationship between oysters and *Pseudomonas* is unknown, and whether *Pseudomonas* is an intermediate player in oyster-induced *R. venosa* metamorphosis remains to be determined.

5. Conclusions

This study revealed the impacts of induction by juvenile oysters on the food habit transition and metamorphosis of *R. venosa*. Significant alterations in critical genes in the digestive system and neuroendocrine system induced by juvenile oysters were observed in competent larvae. Evident alterations in the symbiotic microbiota and host metabolic profiles due to induction by juvenile oysters and development were also identified, and most of these changes were associated with the digestive system and neuroendocrine system. According to these changes, we propose a hypothetical model of juvenile oyster-induced metamorphosis in *R. venosa* in which the compounds released by juvenile oysters are likely to interact with the larvae via taste or olfactory receptors, which trigger neuroendocrine system changes, including CCK and CCK receptor, 5-HT and 5-HT receptor, and NO and NOS. Then, CCK may regulate the secretion of digestive enzymes and the development of the digestive system, which initiates the food habit transition from herbivorous to carnivorous. On the other hand, 5-HT and NOS further regulate the metamorphosis of *R. venosa*, and symbiotic microbiota may also play an important role in affecting the food habit transition and metamorphosis. However, what is released from juvenile oysters and what types of receptors act on larvae need to be further studied. Our results will provide new insight into the regulatory mechanism of metamorphosis in carnivorous gastropods induced by food, which may further reveal the collecting effect of juvenile oyster reefs on *R. venosa* in the natural environment.

Ethics approval and consent to participate

All procedures involved in animal collection, rearing and dissection were conducted following the Guideline of Ethical Regulations

of Animal Welfare of the Institute of Oceanology, Chinese Academy of Sciences (IOCAS 2013.3). Our study protocols were approved by the Animal Welfare Committee of the IOCAS.

Conflict of interest

The authors report no conflict of interest.

Author statement

TZ conceived and designed the experiments. M-JY conducted the experiments. M-JY and HS analyzed the data. JF, Z-LY, PS, JL, ZH, CZ, and X-LW contributed reagents, materials, and analytical tools. M-JY wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.csbj.2021.11.041>.

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